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A. TITLE PAGE

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INSTITUTION	Tuskegee Institute, Alabama
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B. SUMMARY OF PROGRESS

Studies on the hemolysins of Listeria monocytogenes have shown these to be protein in nature, with the relative electrophoretic mobility of gamma type globulins, and are antigenic in the rabbit.

The amount of hemolysin produced was dependent on the strain, the time and temperature of incubation, and the state of the medium (broth or solid). Common hydrocolloids added to the broth medium increased the titer of the hemolysins produced and prevented their early deterioration. The hemolysins are oxygen sensitive and heat labile.

While inducing no detectable toxicity in the intact mice or skin necrosis in the rabbit, the hemolysins appear to exert cytolytic effect on isolated mouse peritoneal exudate cells.

An investigation into the nature of avirulence, indicate the probable role of cytotoxic factor/s, produced during growth in the host, on the establishment of infection and the production of overt disease.

A highly toxic polysaccharide has been isolated from some strains of Listeria monocytogenes and is presently under study.

C. STATEMENT OF PROGRESS

1. Publications

The following articles have either been published, are in press or under preparation:

1. Njoku-Obi, A. N., and E. Jenkins: Quantitative Aspects and Nature of Soluble Hemolysins of Listeria monocytogenes, Bact. Proc., 1962.
2. Osebold, J. W., and A. N. Njoku-Obi: Resistance Mechanisms in Listeriosis. 2nd International Symposium on Listeriosis, Montana, August 29 - 31, 1962.

3. Njoku-Obi, A. N.: Serological Aspects of Listeriosis; The Antigen-Fixation Test. 2nd International Symposium on Listeriosis, Montana, August 29--31, 1962.

4. Njoku-Obi, A. N., and E. Jenkins: Studies on the Soluble Hemolysins of Listeria monocytogenes. Submitted for publication--J. Bacteriol.

5. Njoku-Obi, A. N., Adams, E. W., and K. M. Das: The Nature of Avirulence in Listeria monocytogenes. I. Behavior of Virulent and Avirulent strains in mice. In preparation.

6. Njoku-Obi, A. N., and B. Robinson: The Nature of Avirulence in Listeria monocytogenes. II. Isolation of a toxic polysaccharide. In preparation

2. Hemolysins of Listeria monocytogenes.

Although it has been known for a long time that Listeria monocytogenes produces beta-lysis on blood agar plates and that this hemolysin is soluble and filterable, no information is available on its distribution, nature and role in listeriosis.

In our search for toxins of this organism we turned attention to obtain some information on the hemolysins. One hundred and fifty-seven strains were studied. These strains were obtained from all over the globe including Japan, Eastern Europe, Western Europe, Australia and South America.

The soluble hemolysins were obtained from either Brain Heart Infusion Broth culture supernates or saline extracts of Brain Heart Infusion Agar cultures. The amount of hemolysin produced varied between strains from 0 to 1:4096 H. U., and within some strains from one run to another. Ten per cent of the strains produced no detectable hemolysins and about 6% produced very high titered hemolysins in the range of 1:1024 to 1:4096.

It was found that the strains produced higher titered hemolysins on agar cultures than in broth. The addition of some common hydrocolloids--furcelleran,

cellulose gum and sodium alginate at concentrations of 0.1 to 0.3% to the broth cultures prevented the early deterioration of the hemolysins and induced the yield of higher titers. The time and temperature of incubation affected the amount of hemolysin produced. Thus oxygen was also found to inactivate the hemolysins although not to the extent that has been reported for streptolysin "O." $Na_2S_2O_4$ did reactivate such inactivated hemolysins sometimes to their original titers. Because of this and also because hydrocolloids were found to prevent deterioration and potentiate the titers of the hemolysins, it was thought that incubation under CO_2 might be beneficial. The result of such experiments was contrary to expectation since, many strains failed to produce any under 5% CO_2 incubation and hemolysins produced by the few were of very low titers as compared to incubation in air. The mode of action of the hydrocolloids therefore is speculative. They may act to bind some toxic medium ingredients thus allowing more hemolysins to be produced or to loosely absorb the hemolysins thereby protecting them from the deteriorating influence of oxygen or other oxidizing substances. Their role in increasing the viscosity of the medium and thus minizing O_2 content can't be over looked.

Filtration of the hemolysins through Seitz, Selas and Sintered glass filters inactivated them. This has only been prevented by filtration through millipore under positive pressure. The titers of the hemolysins were also decreased by storage and completely eliminated by heating at 60 degrees Centigrade for 15-30 minutes.

Three hemolysis-rate patterns were noted: maximum titers attained

(1) within 30 minutes at 20-25° Centigrade
(2) after 2 hours at 37° Centigrade and
(3) after 2 hours at 37° Centigrade followed

by over night incubation at 10° Centigrade. Approximately 6% of the strains produced hemolysins that showed pattern (1) hemolysis; 64% showed pattern (2) and 30% pattern (3). In view of recent reports of production of

beta-conditioning factors by some strains of Listeria monocytogenes, pattern (3) might be analogus to the so called hot-cold lysins of the Staphylococcus. It has not yet been determined whether these patterns of hemolysis indicate fundamental differences or not.

No definite correlation could be made with virulence. Some avirulent strains produced rather high titered hemolysins.

Washed red blood cells of the sheep, cow, horse, guinea pig and human were equally acted upon. The use of anti-hemolysin titration for serological diagnosis seems to be clouded by the fact that the normal sera of the species mentioned above were anti-hemolytic in very high dilutions.

In pathogenesis, the role of the hemolysins is not defined. No toxicity could be demonstrated when mice were injected i.v. or i.p. with them. Further no skin necrosis could be elicited in the rabbit. Nevertheless, it is quite probable that the hemolysins do play a part in the intracellular events following phagocytosis. Indirect evidence has been obtained by incubating purified hemolysins with phagocytic cells from the mouse. With washed normal cells, the population was drastically reduced to 15-20% of the original in 2 hours at 37° Centigrade. Cells obtained from mice previously immunized with sub-lethal doses of virulent Listeria organisms, show a much greater resistance to this cytolytic effect. Ninety-two per cent of the original cell population remained and were viable after two hours, under identical conditions. It is speculated that the quite extensive lysis of normal sheep peritoneal exudate cells by Listeria monocytogenes in tissue culture might perhaps be due to the action of the hemolysins. In that experiment, immune cells in immune sera were resistant to this cytolylic action.

The hemolysins are definitely proteins. They are easily precipitated out of solution with Ammonium Sulfate, non-dialyzable, and behaved electrophoretically with the relative mobility of gamma type globulin. They are highly antigenic in the rabbit.

Part of this study was presented at the Annual Meeting of the American Society for Microbiology at Kansas City, Mo., 1962.

A detailed manuscript has been submitted for publication.

3. The Factors Involved in Virulence:

In an earlier study in sheep, it was found that injection of large doses of live avirulent Listeria organisms, in an attempt at immunization, not only failed to confer any protection but also induced no appreciable rise in antibody titer. Contrary to the above, sub-lethal doses of virulent live organisms, similarly inoculated, did significantly protect against intracarotid challenge and induced specific antibody production.

In view of those findings, it was thought necessary to determine, if possible, the nature of avirulence in Listeria monocytogenes. It is well accepted that avirulence may reflect relative inability of the organism to cause a distinct clinical disease in a given host and that this might be manifested by either a relative inability to establish an infection, to accumulate in the tissues and/or to elaborate substances injurious to the host. The studies conducted with mice, aimed to determine which of these factors or combinations thereof operated in listeriosis.

Graded doses of avirulent strain #7648 and virulent #4-52 were injected s. c., i. p. and i. v. in mice. At various intervals two mice in each group were killed and bacterial counts made of the following: liver, spleen, lymph nodes, lungs, peritoneal washing (where applicable), heart blood, heart and brain. A third mouse was used for histopathology. The following results were obtained:

1. Persistence in tissues:

A. AVIRULENT ORGANISMS

With small doses of the avirulent strain (10^3, 10^5), the bacteria were rapidly cleared out of the system. By the second day less than

10 organisms were found in any tissue counted.

When the dose was large (2×10^9), a different picture was observed. As was expected the number of organisms/organ steadily decreased. We, however, did not expect that the organisms persisted in the tissues up to the ninth day on the average of approximately 10^3 organisms/organ. This perhaps might indicate the slowing of the bacteriacidal mechanisms by the large number of organisms injected.

Histologically, no cellular damage was observed with the smaller doses of 10^3 and 10^5 organisms. Even with the heavy inoculum of 2×10^9, only slight reactions of the liver cells, few foci of mononuclear infiltration and perivascular cuffings, were noted. On the 5th day a slight necrosis of the hepatic cord cells were seen.

None of the mice injected with 2×10^9 of the avirulent organisms died after four weeks.

B. VIRULENT ORGANISMS:

The virulent organism had an LD_{50} of 10^6 cells for mice. The sizes of inocula employed were 10^2, 10^4, 10^7 cells.

The smallest inoculum was quickly removed from the tissues. The median dose, did show persistence up to the 4th day. There was however, no evidence of multiplication and none of the mice died after 3 weeks. At that dosage level evidences of tissue cell destruction were noted. The lymph nodes (inguinae), liver and spleen were the organs most affected when the injection was via the s. c. route. There were marked depletion of germinal centers and edema in both the lymph nodes and spleen at 24 hours, followed on about the 5th day with hyperplasia of R. E. cells in those organs. This probably indicated antibody production. In the liver, the sequence of events was neutrophilic accumulations and edema, mononuclear infiltration and slight paracentral necrosis.

The highest inoculum of 10^7 cells presented a different picture. The number of organisms/organ increased logarithmically until all the mice died 5 to 7 days later.

Histopathologically, at 24 hours after inoculation greyish white necrotic spots, 1-2 mm in diameter, appeared over the surface of the liver. These increased in number with time and by the 5th day the whole liver was covered by them. Gross lesions on the spleen were evident at the 72nd hour. These also increased in number with time but were not as numerous as on the liver. No gross lesions were seen on other organs.

Microscopically, the liver was the most vulnerable organ. Cellular necrosis was evident at the 24th hour and progressed with time. The necrosis was of the caseous type with a thick rim of leucocytic accumulation; it appeared to start around the branches of the portal vein and bile ducts in the portal areas. By the 4th day, it has spread extensively so that each lobule had one or more foci of necrosis. At the 6th day approximately 75% of the liver was dead. Bacteria were numerous in the necrotic areas.

Next in order of involvement was the spleen. At 24 hours, the sinusoids were engorged and neutrophiles were abundant in the red pulp. At 48 hours, increased cellular debris was observed in the spleenic corpuscles. Macrophages began to replace neutrophiles. Definite necrosis became evident on the third day. The necrosis was of the caseation type with an outer zone of disintegrated neutrophiles. This was first noticed in the red pulp below the capsule and from there, necrosis gradually extended into the depth of the organ. The reticulum of the red pulp, the sinusoids and the spleenic corpuscles were involved in necrosis, in that descending order.

The above described phase of the study was shown that to establish an infection large numbers of Listeria monocytogenes (virulent) was needed and pointed to the possible role of some "cytotoxic factor" elaborated by this

organism which induced both cellular reaction and cell death.

In the second phase of the study, the results obtained seem to support the role of this postulated cytotoxic factor. When allowed to accumulate in the tissues by the administration of perdnisolone (25 mg. per mouse) twenty-four hours before inoculation of bacteria and 48 hours after, the avirulent organisms multiplied and killed the test animals within 5 days. Similar results were obtained when stress was induced by fasting.

The pathology was very similar to that caused by the virulent organisms. It was however less both in severity and intensity. When compared with the avirulent strain alone, the lesions produced by the avirulent strain plus cortisone was more severe and extensive. These reactions would indicate either the presence of a very small number of virulent bacteriae cells in the large population of avirulent bacteria injected (2×10^9), which in a host under stress, with suppressed clearance and other defensive mechanisms, were able to establish and multiply extensively in the excellent nutritive internal meliew of the host; or that like the virulent strain, the avirulents do also produce the postulated "cytotoxic factor" to a very much less extent, which could accumulate as the avirulents established and multiplied extensively <u>in situ</u> under the influence of cortisone. Also both may operate at the same time.

The first hypotheses seems to be supported by the following results tabulated below.

The Effect of Cortisone on Graded doses of Avirulent and Virulent Listeria monocytogenes.

No. organism injected	Avirulent Alone	Avirulent +cortisone	Virulent Alone	Virulent +cortisone
2×10^9	0/3*	3/3	3/3	3/3
2×10^8	0/3	3/3	2/3	3/3
2×10^7	N.D	2/3	2/3	3/3
2×10^6	N.D.	0/3	1/3	3/3
2×10^5	N.D.	0/3	0/3	3/3
2×10^4	N.D.	0/3	0/3	2/3
2×10^3	N.D.	0/3	0/3	2/3

*= No. of mice dead/No. challenged.

1 = 2.5 mg. prednisolone/mouse, 24 hours before and 48 hours after challenge.

It would appear that the avirulent population contained less than 2000 organisms capable of establishing infection and kill the mice. The potentiation of the virulence of the "virulent" strain by cortisone is very remarkable.

Two main areas of study are at the present being pursued. One of these, is the effect of immunization on the persistence and infectivity; and the other is the nature of this cytotoxic factor.

Preliminary experiments to explore the first of the problems have been very encouraging. Thus, mice immunized with virulent organisms and challanged later with 2×10^9 avirulent organisms both s. c. and i. p., cleared the organisms in less than 24 hours with counts of less than 10 organisms/tissue. It is to be recollected that in normal mice, these organisms persisted in some cases up to 9 days with about 100 to 1000 organisms/tissue.

With the virulent organisms, the clearance mechanisms was also greatly activated. Whereas in the normal mice, these organisms multiplied logarithmically and killed all the mice in about 5 days, they were effectively eliminated from the immune mice in 48 hours with no significant pathology manifested. Furthermore, whereas in the normal mice, with both virulent and avirulent organisms, bacteria were cultured from the brain as early as 4 hours after inoculation, no such bacteria could be isolated from the brain of any of the immune mice. From the pattern of lesions described earlier in the normal mice, the spread of organisms appeared to be hematogenous. Through this route the brain could have received some showering. That no organisms could be found in the immunized mice, challenged with either virulent or avirulent organisms, would indicate activation of humoral and cellular defense mechanisms and could explain the prevention of encephalitis in sheep immunized with virulent organisms.

Re the "cytotoxic factor", attempts to demonstrate this with culture filterates of virulent organisms have proved abortive. It was possible however, to isolate a highly toxic factor from Listeria monocytogenes. This factor was obtained by phenol extraction after the method of Westphal. The phenol insoluble fraction, which was Biuret negative and Molisch positive, killed mice with 10-15 minutes after i. v. injection of about 0.01 mg. Studies are in progress to determine relative distribution of this agent in virulent and avirulent organisms, its chemical composition and pharmacodynamic action.

Immunity in listeriosis seems to depend not only on the number of organisms present in the host but most importantly on the production of certain essential antigens inside the host. The avirulent organisms were found to persist in the mice up to 9 days when about 2×10^9 cells were inoculated. This would have normally given adequate opportunity for the immune mechanisms to be activated, but yet the animals behaved as normal, non-immunized animals.

On the other hand, 1 X 10^4 virulent organisms did not persist as long in the mice but from clearance studies as well as challenge, the behavior of the mice appeared to be very much altered. On this, the proposed studies of the antigens of virulent and avirulent organisms and the in vivo cultivation of listerial organisms for immunization might shed some further light.

The results obtained in these studies are being readied for publication.

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